ISOLATED NUCLEIC ACIDS COMPRISING LISTERIAL DAL AND DAT GENES

GOVERNMENT SUPPORT

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FIELD OF THE INVENTION

The invention relates to vaccine vectors comprising bacteria.

BACKGROUND OF THE INVENTION

The use of vaccines is a cost-effective medical tool for the management of infectious diseases, including infectious diseases caused by bacteria, viruses, parasites, and fungi. In addition to effecting protection against infectious diseases, vaccines may now also be developed which stimulate the host's immune system to intervene in tumor growth.

Host immune responses include both the humoral immune response involving antibody production and the cell-mediated immune response. Protective immunization via vaccine has usually been designed to induce the formation of humoral antibodies directed against infectious agents, tumor cells, or the action of toxins. However, the control of certain diseases characterized by the presence of tumor cells or by chronic infection of cells with infectious agents, often requires a cell-mediated immune response either in place of, or in addition to the generation of antibody. While the humoral immune response may be induced using live infectious agents and agents which have been inactivated, a cellular immune response is most effectively induced through the use of live agents as vaccines. Such live agents include live infectious agents which may gain access to the cytoplasm of host cells where the

proteins encoded by these agents are processed into epitopes which when presented to the cellular immune system, induce a protective response.

Microorganisms, particularly *Salmonella* and *Shigella* which have been attenuated using a variety of mechanisms, have been examined for their ability to encode and express heterologous antigens (Coynault et al., 1996, Mol. Microbiol. 22:149-160; Noriega et al., 1996, Infect. Immun. 64:3055-3061; Brett et al., 1993, J. Immunol. 150:2869-2884; Fouts et al., 1995, Vaccine 13:1697-1705, Sizemore et al., 1995, Science 270:299-302). Such bacteria may be useful as live attenuated bacterial vaccines which serve to induce a cellular immune response directed against a desired heterologous antigen.

Listeria monocytogenes (*L. monocytogenes*) is the prototypic intracellular bacterial pathogen which elicits a predominantly cellular immune response when inoculated into an animal (Kaufmann, 1993, Ann. Rev. Immunol. 11:129-163). When used as a vector for the transmission of genes encoding heterologous antigens derived from infectious agents or derived from tumor cells, recombinant *Listeria* encoding and expressing an appropriate heterologous antigen have been shown to successfully protect mice against challenge by lymphocytic choriomeningitis virus (Shen et al., 1995, Proc. Natl. Acad. Sci. USA 92:3987-3991; Goossens et al., 1995, Int. Immunol. 7:797-802) and influenza virus (Ikonomidis et al., 1997, Vaccine 15:433-440). Further, heterologous antigen expressing recombinant *Listeria* have been used to protect mice against lethal tumor cell challenge (Pan et al., 1995, Nat. Med. 1:471-477; Paterson and Ikonomidis, 1996, Curr. Opin. Immunol. 8:664-669). In addition, it is known that a strong cell-mediated immune response directed against HIV-1 gag protein may be induced in mice infected with a recombinant *L. monocytogenes* comprising HIV-1 *gag* (Frankel et al., 1995, J. Immunol. 155:4775-4782).

Although the potential broad use of *Listeria* as a vaccine vector for the prevention and treatment of infectious disease and cancer has significant advantages over other vaccines, the issue of safety during use of *Listeria* is not trivial. The use of the most common strain of *Listeria, L. monocytogenes*, is accompanied by potentially

severe side effects, including the development of listeriosis in the inoculated animal. This disease, which is normally food-borne, is characterized by meningitis, septicemia, abortion and often a high rate of mortality in infected individuals. While natural infections by *L. monocytogenes* are fairly rare and may be readily controlled by a number of antibiotics, the organism may nevertheless be a serious threat in immunocompromised or pregnant patients. One large group individuals that might benefit from the use of *L. monocytogenes* as a vaccine vector are individuals who are infected with HIV. However, because these individuals are severely immunocompromised as a result of their infection, the use of *L. monocytogenes* as a vaccine vector is undesirable unless the bacteria are fully and irreversibly attenuated.

There is a need for the development of a strain of *L. monocytogenes* for use as a vaccine in and of itself and for use as a bacterial vaccine vector which is attenuated to the extent that it is unable to cause disease in an individual into whom it is inoculated. The present invention satisfies this need.

SUMMARY OF THE INVENTION

The invention includes a method of eliciting a T cell immune response to an antigen in a mammal comprising administering to the mammal an auxotrophic attenuated strain of *Listeria* which expresses the antigen, wherein the auxotrophic attenuated strain comprises a mutation in at least one gene whose protein product is essential for growth of the *Listeria*. In a preferred embodiment, the *Listeria* is *L. monocytogenes*. In another preferred embodiment, the auxotrophic attenuated strain is auxotrophic for the synthesis of D-alanine. In addition, the mutation comprises a mutation in both the *dal* and the *dat* genes of the *Listeria*.

In one aspect of the invention, the auxotrophic attenuated strain further comprises DNA encoding a heterologous antigen, or the the auxotrophic attenuated strain further comprises a vector comprising a DNA encoding a heterologous antigen. The heterologous antigen may be an HIV-1 antigen.

The invention also includes a vaccine comprising an auxotrophic attenuated strain of *Listeria* which expresses an antigen, wherein the auxotrophic attenuated strain comprises a mutation in at least one gene whose protein product is essential for growth of the *Listeria.*

In preferred embodiments, the *Listeria* is *L. monocytogenes*. In other preferred embodiments, the auxotrophic attenuated strain is auxotrophic for the synthesis of D-alanine. In yet other preferred embodiments, the mutation comprises a mutation in both the *dal* and the *dat* genes of the *Listeria.*

The auxotrophic attenuated strain may further comprise DNA encoding a heterologous antigen or a vector comprising a DNA encoding a heterologous antigen.

The heterologous antigen may be an HIV-1 antigen.

Also included in the invention is an isolated nucleic acid sequence comprising a portion of a *Listeria dal* gene and an isolated nucleic acid sequence comprising a portion of a *Listeria dat* gene.

In addition, the invention includes an isolated strain of *Listeria* comprising a mutation in a *dal* gene and a mutation in a *dat* gene which render the strain auxotrophic for D-alanine. In one aspect, the isolated strain of *Listeria* further comprises a heterologous antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the DNA sequence of the *L. monocytogenes* alanine racemase gene (*dal*) of *L. monocytogenes* and the amino acid sequence encoded thereby. The lysyl residue involved in the binding of pyridoxal-P is indicated by an asterisk.

Figure 2 depicts the linear alignment of the deduced amino acid sequences of the alanine racemases of *L. monocytogenes* (LMDAL), *B. stearothermophilus*, (BSTDAL), and *B. subtilis* (BSUBDAL). Identical amino acids are boxed.

Figure 3 is the DNA sequence of the *L. monocytogenes* D-amino acid aminotransferase gene (*dat*) and the amino acid sequence encoded thereby. The lysyl residue involved in the binding of pyridoxal-P is indicated by an asterisk.

Figure 4 depicts the linear alignment of the deduced amino acid sequences of the D-amino acid aminotransferases of *L. monocytogenes* (LMDAT), *S. haemolyticus* (SHAEDAT), *B. sphaericus* (BSPHDAT), and *Bacillus* species YM-1 (BSPDAT). Identical amino acids are boxed.

Figure 5 is a graph depicting the growth requirement for D-alanine of the *dal*⁻*dat*⁻ double mutant strain of *L. monocytogenes*. The *dal*⁻*dat*⁻ (daldat) and wild-type (*L. monocytogenes*+) strains of *L. monocytogenes* were grown in liquid culture in BHI medium at 37°C in the presence (+D-ala) or absence (-D-ala) of exogenous D-alanine (100 μg/ml). In additional experiments, the mutant cell culture was also provided D-alanine after 30 minutes and after 60 minutes.

Figure 6 is a series of images of light micrographs depicting the growth of wild-type *L. monocytogenes* (Panel A) and the *dal*⁻*dat*⁻ double mutant strain of *L. monocytogenes* (Panel B) in J774 macrophages at 5 hours after infection with about 5 bacteria per mouse cell. Panel C illustrates an infection by double mutant bacteria in the continuous presence of D-alanine (80 μg/ml). Arrowheads point to some mutant bacteria.

Figure 7 is a series of graphs depicting infection of mammalian cells with the *dal*⁻*dat*⁻ double mutant (open circles) and wild-type strains of *L. monocytogenes* (closed circles). Mammalian cells which were infected included J774 murine macrophage-like cells (Panel A), primary murine bone marrow macrophages (Panel B), and human epithelial cells (HeLa) (Panel C). Panel A also depicts mutant infection in the presence of D-alanine (100 μg/ml) (closed squares) and in the presence of D-alanine from 0 to 4 hrs during infection (open squares).

Figure 8 is a series of images of photomicrographs depicting the association of actin with intracytoplasmic wild-type *L. monocytogenes* (Panel A: 2 hours; Panel B: 5 hours) or with the *dal*⁻*dat*⁻ double mutant of *L. monocytogenes* (Panel

C: 2 hours wherein D-alanine was present from 0 to 30 minutes; Panel D: 5 hours, wherein D-alanine was present from 0 to 30 minutes; Panel E: 5 hours, wherein D-alanine was present continuously), following infection of J744 cells with these bacteria. The images on the top row illustrate the binding of FITC-labeled anti-Listerial antibodies to total bacteria, while the bottom row illustrates the binding of TRITC-labeled phalloidin to actin. The arrowheads point to some bacteria associated with actin.

Figure 9 is a graph depicting the protection of BALB/c mice against challenge with ten times the LD_{50} of wild-type *L. monocytogenes* by immunization with the *dal dat* double mutant strain of *L. monocytogenes*. Groups of 5 mice were immunized with the following organisms: (1) 4 x 10^2 wild-type *L. monocytogenes*, (2) 2 x 10^7 *dal dat* (+D-alanine supplement), (3) 2 x 10^5 *dal dat* (+D-alanine supplement), (4) 2 x 10^4 *dal dat* (+D-alanine supplement), (5) 2 x 10^2 *dal dat* mutant *dal dat* (no D-alanine supplement). Mice were challenged 21-28 days after immunization. Log_{10} protection was calculated as described in the Examples.

Figure 10 is a graph depicting the recovery of bacteria from spleens of BALB/c mice following sublethal infection with wild type *L. monocytogenes* (closed circles), the *dal dat* mutant in the absence of D-alanine (open circles), and the *dal dat* mutant in the presence of 20 mg D-alanine (open squares). The points at day 0 illustrate the total number of organisms injected, not the number of bacteria per spleen.

Figure 11 is a series of graphs depicting the cytolytic activity of splenocytes isolated from mice at 10-14 days after infection with in Figure 11A, wild type *L. monocytogenes* (●○), or niave control (■□). Figure 11B, *dal dat* mutant: 3 x 10^7 bacteria (△); 3 x 10^7 bacteria with boost at 10 days (△); 3 x 10^7 bacteria wherein animals were provided D-alanine subcutaneously (●○); 3 x 10^7 bacteria plus 2 mg/ml D-alanine (■) or 0.2 mg/ml D-alanine in drinking water (▲).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to vaccines comprising attenuated strains of *Listeria*, wherein the bacteria have been attenuated by the introduction of auxotrophic mutations in the *Listeria* genomic DNA. These strains are herein referred to as attenuated auxotrophic strains or "AA strains" of *Listeria*.

It has been discovered in the present invention that the administration of an AA strain of *Listeria* to a mammal results in the development of a host cytotoxic T cell (CTL) response directed against *Listeria* following survival of the AA strain in the mammal for a time sufficient for the development of the response. The AA strain provides protection against challenge by *L. monocytogene* and is therefore suitable for use in a vaccine for protection against infection by this organism. The AA strain of the invention may thus be employed as a vaccine for the prevention and/or treatment of infection by *Listeria*. In addition, the AA strain of the invention may have added to it a heterologous gene wherein the gene is expressed by the AA strain. Such AA strains encoding additional heterologous genes are useful as bacterial vector vaccines for the prevention and/or treatment of infection caused by any number of infectious agents and for the prevention and/or treatment of tumors in mammals.

AA strains of *Listeria* that are auxotrophic for D-alanine are contemplated as part of this invention.

By the term "auxotrophic for D-alanine", as used herein, is meant that the AA strain of *Listeria* is unable to synthesize D-alanine in that it cannot grow in the absence of D-alanine and therefore requires exogenously added D-alanine for growth.

D-alanine is required for the synthesis of the peptidoglycan component of the cell wall of virtually all bacteria, and is found almost exclusively in the microbial world. Wild-type *Listeria* species synthesize D-alanine and thus do not require exogenously added D-alanine for growth. An AA strain of *L. monocytogenes* has been discovered in the present invention which is unable to synthesize D-alanine. This organism may be grown in the laboratory but is incapable of growth outside the laboratory in unsupplemented environments, including in the cytoplasm of eukaryotic

host cells, the natural habitat of this organisms during infection. Such strains of *Listeria* are useful as vaccines.

By the term "vaccine," as used herein, is meant a population of bacteria which when inoculated into a mammal has the effect of stimulating a cellular immune response comprising a T cell response. The T cell response may be a cytotoxic T cell response directed against macromolecules produced by the bacteria. However, the induction of a T cell response comprising other types of T cells by the vaccine of the invention is also contemplated. For example, *Listeria* infection also induces both CD4+ T cells and CD8+ T cells. Induced CD4+ T cells are responsible for the synthesis of cytokines, such as interferon-γ, IL-2 and TNF-α. CD8+ T cells may be cytotoxic T cells and also secrete cytokines such as interferon-γ and TNF-α. All of these cells and the molecules synthesized therein play a role in the infection and subsequent protection of the host against *Listeria*. Cytokines produced by these cells activate additional T cells and also macrophages and recruit polymorphonuclear leukocytes to the site of infection.

Both prophylactic and therapeutic vaccines are contemplated as being within the scope of the present invention, that is, vaccines which are administered either prior to or subsequent to the onset of disease are included in the invention.

D-alanine auxotrophic mutants useful as vaccine vectors may be generated in a number of ways. As described in the Examples presented herein, disruption of one of the alanine racemase gene (*dal*) or the D-amino acid aminotransferase gene (*dat*), each of which is involved in D-alanine synthesis, did not result in a bacterial strain which required exogenously added D-alanine for growth. However, disruption of both the *dal* gene and the *dat* gene generated an AA strain of *Listeria* which required exogenously added D-alanine for growth.

The generation of AA strains of *Listeria* deficient in D-alanine synthesis may be accomplished in a number of ways that are well known to those of skill in the art, including deletion mutagenesis, insertion mutagenesis, and mutagenesis which results in the generation of frameshift mutations, mutations which effect premature

termination of a protein, or mutation of regulatory sequences which affect gene expression. Mutagenesis can be accomplished using recombinant DNA techniques or using traditional mutagenesis technology using mutagenic chemicals or radiation and subsequent selection of mutants. Deletion mutants are preferred because of the accompanying low probability of reversion of the auxotrophic phenotype. Mutants of D-alanine which are generated according to the protocols presented herein may be tested for the ability to grow in the absence of D-alanine in a simple laboratory culture assay. Those mutants which are unable to grow in the absence of this compound are selected for further study.

In addition to the aforementioned D-alanine associated genes, other genes involved in D-alanine synthesis may be used as targets for mutagenesis of *Listeria*. Such genes include, but are not limited to any other known or heretofore unknown D-alanine associated genes.

Genes which are involved in the synthesis of other metabolic components in a bacterial cell may also be useful targets for the generation of attenuated auxotrophic mutants of *Listeria*, which mutants may also be capable of serving as bacterial vaccine vectors for use in the methods of the present invention. The generation and characterization of such other AA strains of *Listeria* may be accomplished in a manner similar to that described herein for the generation of D-alanine deficient AA strains of *Listeria*.

Additional potential useful targets for the generation of additional auxotrophic strains of *Listeria* include the genes involved in the synthesis of the cell wall component D-glutamic acid. To generate D-glutamic acid auxotrophic mutants, it is necessary to inactivate the *dat* gene, which is involved in the conversion of D-glu + pyr to alpha-ketoglutarate + D-ala and the reverse reaction. It is also necessary to inactivate the glutamate racemase gene, *dga*. Other potential useful targets for the generation of additional auxotrophic strains of *Listeria* are the genes involved in the synthesis of diamimopimelic acid. In this instance, a gene encoding aspartate beta-

semialdehyde dehydrogenase may be inactivated (Sizemore et al., 1995, Science 270:299-302).

By the term "attenuation," as used herein, is meant a diminution in the ability of the bacterium to cause disease in an animal. In other words, the pathogenic characteristics of the attenuated *Listeria* strain have been lessened compared with wild-type *Listeria*, although the attenuated *Listeria* is capable of growth and maintenance in culture. Using as an example the intravenous inoculation of Balb/c mice with an attenuated *Listeria*, the lethal dose at which 50% of inoculated animals survive (LD_{50}) is preferably increased above the LD_{50} of wild-type *Listeria* by at least about 10-fold, more preferably by at least about 100-fold, more preferably at least about 1,000 fold, even more preferably at least about 10,000 fold, and most preferably at least about 100,000-fold. An attenuated strain of *Listeria* is thus one which does not kill an animal to which it is administered, or is one which kills the animal only when the number of bacteria administered is vastly greater than the number of wild type non-attenuated bacteria which would be required to kill the same animal. An attenuated bacterium should also be construed to mean one which is incapable of replication in the general environment because the nutrient required for its growth is not present therein. Thus, the bacterium is limited to replication in a controlled environment wherein the required nutrient is provided. The attenuated strains of the present invention are therefore environmentally safe in that they are incapable of uncontrolled replication.

It is believed that any *Listeria* species capable of infectious disease may be genetically attenuated according to the methods of the present invention to yield a useful and safe bacterial vaccine, provided the attenuated *Listeria* species exhibits an LD_{50} in a host organism that is significantly greater than that of the non-attenuated wild type species. Thus, strains of *Listeria* other than *L. monocytogenes* may be used for the generation of attenuated mutants for use as vaccines. Preferably, the *Listeria* strain useful for the generation of attenuated vaccines is *L. monocytogenes*.

An AA strain of *Listeria* may be generated which encodes and expresses a heterologous antigen. The heterologous antigen encoded by the AA strain of *Listeria*

is one which when expressed by *Listeria* is capable of providing protection in an animal against challenge by the infectious agent from which the heterologous antigen was derived, or which is capable of affecting tumor growth and metastasis in a manner which is of benefit to a host organism. Heterologous antigens which may be introduced into an AA strain of *Listeria* by way of DNA encoding the same thus include any antigen which when expressed by *Listeria* serves to elicit a cellular immune response which is of benefit to the host in which the response is induced. Heterologous antigens therefore include those specified by infectious agents, wherein an immune response directed against the antigen serves to prevent or treat disease caused by the agent. Such heterologous antigens include, but are not limited to, viral, bacterial, fungal or parasite surface proteins and any other proteins, glycoproteins, lipoprotein, glycolipids, and the like. Heterologous antigens also include those which provide benefit to a host organism which is at risk for acquiring or which is diagnosed as having a tumor. The host organism is preferably a mammal and most preferably, is a human.

By the term "heterologous antigen," as used herein, is meant a protein or peptide, a glycoprotein or glycopeptide, a lipoprotein or lipopeptide, or any other macromolecule which is not normally expressed in *Listeria*, which substantially corresponds to the same antigen in an infectious agent, a tumor cell or a tumor-related protein. The heterologous antigen is expressed by an AA strain of *Listeria*, and is processed and presented to cytotoxic T-cells upon infection of mammalian cells by the AA strain. The heterologous antigen expressed by *Listeria* species need not precisely match the corresponding unmodified antigen or protein in the tumor cell or infectious agent so long as it results in a T-cell response that recognizes the unmodified antigen or protein which is naturally expressed in the mammal.

By the term "tumor-related antigen," as used herein, is meant an antigen which affects tumor growth or metastasis in a host organism. The tumor-related antigen may be an antigen expressed by a tumor cell, or it may be an antigen which is

expressed by a non-tumor cell, but which when so expressed, promotes the growth or metastasis of tumor cells.

The types of tumor antigens and tumor-related antigens which may be introduced into *Listeria* by way of incorporating DNA encoding the same, include any known or heretofore unknown tumor antigen.

The heterologous antigen useful in vaccine development may be selected using knowledge available to the skilled artisan, and many antigenic proteins which are expressed by tumor cells or which affect tumor growth or metastasis or which are expressed by infectious agents are currently known. For example, viral antigens which may be considered as useful as heterologous antigens include but are not limited to the nucleoprotein (NP) of influenza virus and the gag protein of HIV. Other heterologous antigens include, but are not limited to, HIV env protein or its component parts gp120 and gp41, HIV nef protein, and the HIV pol proteins, reverse transcriptase and protease. In addition, other viral antigens such as herpesvirus proteins may be useful. The heterologous antigens need not be limited to being of viral origin. Parasitic antigens, such as, for example, malerial antigens, are included, as are fungal antigens, bacterial antigens and tumor antigens.

As noted herein, a number of proteins expressed by tumor cells are also known and should be included in the list of heterologous antigens which may be inserted into the vaccine strain of the invention. These include, but are not limited to, the bcr/abl antigen in leukemia, HPVE6 and E7 antigens of the oncogenic virus associated with cervical cancer, the MAGE1 and MZ2-E antigens in or associated with melanoma, and the MVC-1 and HER-2 antigens in or associated with breast cancer.

The introduction of DNA encoding a heterologous antigen into a strain of *Listeria* may be accomplished, for example, by the creation of a recombinant *Listeria* in which DNA encoding the heterologous antigen is harbored on a vector, such as a plasmid for example, which plasmid is maintained and expressed in the *Listeria* species. Alternatively, DNA encoding the heterologous antigen may be stably integrated into the *Listeria* chromosome by employing, for example, transposon

mutagenesis or by homologous recombination. A preferred method for producing recombinant *Listeria* having a gene encoding a heterologous antigen integrated into the chromosome thereof, is the induction of homologous recombination between a temperature sensitive plasmid comprising DNA encoding the antigen and *Listeria* chromosomal DNA. Stable transformants of *Listeria* which express the desired antigen may be isolated and characterized as described herein in the experimental examples. This method of homologous recombination is advantageous in that site directed insertion of DNA encoding the heterologous antigen is effected, thereby minimizing the possibility of disruption of other areas of the *Listeria* chromosome which may be essential for growth of this organism.

Several approaches may be employed to express the heterologous antigen in *Listeria* species as will be understood by one skilled in the art once armed with the present disclosure. Genes encoding heterologous antigens are preferably designed to either facilitate secretion of the heterologous antigen from the bacterium or to facilitate expression of the heterologous antigen on the *Listeria* cell surface.

While the heterologous antigen preferably comprises only a desired antigen along with appropriate signal sequences and the like, also contemplated in the invention is a fusion protein which comprises the desired heterologous antigen and a secreted or cell surface protein of *Listeria*. Listerial proteins which are suitable components of such fusion proteins include, but are not limited to, listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase (PI-PLC). A fusion protein may be generated by ligating the genes which encode each of the components of the desired fusion protein, such that both genes are in frame with each other. Thus, expression of the ligated genes results in a protein comprising both the heterologous antigen and the listerial protein. Expression of the ligated genes may be placed under the transcriptional control of a listerial promoter/regulatory sequence such that expression of the gene is effected during growth and replication of the organism. Signal sequences for cell surface expression and/or secretion of the fused protein may

also be added to genes encoding heterologous antigens in order to effect cell surface expression and/or secretion of the fused protein.

When the heterologous antigen is used alone (i.e., in the absence of fused *Listeria* sequences), it may be advantageous to fuse thereto signal sequences for cell surface expression and/or secretion of the heterologous antigen. The procedures for accomplishing this are well know in the art of bacteriology and molecular biology.

The DNA encoding the heterologous antigen which is expressed in the vaccine strain of the invention must be preceeded by a suitable promoter to facilitate such expression. The appropriate promoter/regulatory and signal sequences to be used will depend on the type of listerial protein desired in the fusion protein and will be readily apparent to those skilled in the art of listeria molecular biology. For example, preferred *L. monocytogenes* promoter/regulatory and/or signal sequences which may be used to direct expression of a fusion protein include, but are not limited to, sequences derived from the *Listeria hly* gene which encodes LLO, the *Listeria* p60 gene (Bouwer et al., 1996, Infect. Immun. 64:2515-2522) and possibly the *Listeria actA* gene which encodes a surface protein necessary for *L. monocytogenes* actin assembly. Other promoter sequences which might be useful in some circumstances include the *plcA* gene which encodes PI-PLC, the listeria *mpl* gene, which encodes a metalloprotease, the listeria *plcB* gene encoding a phospholipase C, and the listeria *inlA* gene which encodes internalin, a listeria membrane protein. For a review of genes involved in *L. monocytogenes* pathogenesis, see Portnoy et al. (1992, Infect. and Immun. 60:1263-1267). It is also contemplated as part of this invention that heterologous regulatory elements such as promoters derived from phage and promoters or signal sequences derived from other bacterial species may be employed for the expression of a heterologous antigen by the *Listeria* species.

Examples of the use of recombinant *L. monocytogenes* strains that express a heterologous antigen for induction of an immune response against tumor cell antigens or infectious agent antigens are described in U.S. Patent Application Nos.

08/366,372 and 08/366,477, respectively. The disclosures of these two patent applications are hereby incorporated herein by reference.

The data presented herein indicate that certain AA strains of *Lis*teria may undergo osmotic lysis following infection of a host cell. Thus, if the *Listeria* which is introduced into the host cell comprises a vector, the vector is released into the cytoplasm of the host cell. The vector may comprise DNA encoding a heterologous antigen. Uptake into the nucleus of the vector DNA enables transcription of the DNA encoding the heterologous antigen and subsequent expression of the antigen in and/or secretion of the same from the infected host cell. Typically, the vector is a plasmid that is capable of replication in *Listeria.* The vector may encode a heterologous antigen, wherein expression of the antigen is under the control of eukaryotic promoter/regulatory sequences. Typical plasmids having suitable promoters that might be employed include, but are not limited to, pCMVbeta comprising the immediate early promoter/enhancer region of human cytomegalovirus, and those which include the SV40 early promoter region or the mouse mammary tumor virus LTR promoter region.

Thus, it is also contemplated as part of the present invention that AA strains of *Listeria* may be employed as a vaccine for the purpose of stimulating a CTL immune response against an infectious agent or a tumor cell, wherein the AA strain comprises a vector encoding a heterologous antigen that may be expressed using a eukaryotic expression system. According to the invention, the vector is propagated in the AA strain of *Listeria* concomitant with the propagation of the AA strain itself. The vector may be, for example, a plasmid that is capable of replication in the AA strain or the vector may be lysogenic phage. The vector must contain a prokaryotic origin of replication and must not contain a eukaryotic origin of replication in order that the vactor is capable of replication in a prokaryotic cell but, for safety reasons, is rendered absolutely incapable of replication in eukaryotic cells.

A cytotoxic T-cell response in a mammal is defined as the generation of cytotoxic T-cells capable of detectably lysing cells presenting an antigen against which the T cell response is directed. Preferably, within the context of the present invention,

the T cell response is directed against a heterologous antigen expressed in an AA strain of *Listeria* or which is expressed by a vector which is delivered to a cell via Listeria infection. Assays for a cytotoxic T-cell response are well known in the art and include, for example, a chromium release assay (Frankel et al., 1995, J. Immunol. 155:4775-4782). In addition to a chromium release assay, an assay for released lactic acid dehydrogenase may be performed using a Cytotox 96 kit obtained from Promega Biotech, WI.

In preferred embodiments and using a chromium release assay, at an effector cell to target cell ratio of about 50:1, the percentage of target cell lysis is preferably at least about 10% above the background level of cell lysis. The background level of cell lysis is the percent lysis of cells which do not express the target antigen. More preferably, the percentage of target cell lysis is at least about 20% above background; more preferably, at least about 40% above background; more preferably, at least about 60% above background; and most preferably, at least about 70% above background.

The vaccines of the present invention may be administered to a host vertebrate animal, preferably a mammal, and more preferably a human, either alone or in combination with a pharmaceutically acceptable carrier. The vaccine is administered in an amount effective to induce an immune response to the *Listeria* strain itself or to a heterologous antigen which the *Listeria* species has been modified to express. The amount of vaccine to be administered may be routinely determined by one of skill in the art when in possession of the present disclosure. A pharmaceutically acceptable carrier may include, but is not limited to, sterile distilled water, saline, phosphate buffered solutions or bicarbonate buffered solutions. The pharmaceutically acceptable carrier selected and the amount of carrier to be used will depend upon several factors including the mode of administration, the strain of *Listeria* and the age and disease state of the vaccinee. Administration of the vaccine may be by an oral route, or it may be parenteral, intranasal, intramuscular, intravascular, intrarectal, intraperitoneal, or any one of a variety of well-known routes of administration. The route of administration

may be selected in accordance with the type of infectious agent or tumor to be treated. The vaccines of the present invention may be administered in the form of elixirs, capsules or suspensions for oral administration or in sterile liquids for parenteral or intravascular administration. The vaccine may also be administered in conjunction with a suitable adjuvant, which adjuvant will be readily apparent to the skilled artisan.

The immunogenicity of the AA strain of the invention may be enhanced in several ways. For example, a booster inoculation following the initial inoculation may be used to induce an enhanced CTL response directed against the AA strain.

In another approach, transient suppression of the auxotrophic phenotype of the AA strain is accomplished by providing the AA strain with the required nutrient for a period of time shortly before, after, or concomitant with administration of the *Listeria* vaccine to the host. The organism will replicate for the brief period during which the nutrient is present, after which, upon exhaustion of the supply of the nutrient, the organism will cease replication. This brief period of controlled replication will serve to provide more organisms in the host in a manner similar to that of natural infection by *Listeria*, which should stimulate an enhanced CTL response directed against the organism and antigens expressed thereby.

In yet another approach, the use of a suicide plasmid may be employed to conditionally suppress the attenuation of the *Listeria* AA strain by temporarily supplying the missing enzyme or enzymes to the bacterium for synthesis of the essential nutrient. A suitable suicide plasmid includes pKSV7, the same plasmid which was used to mediate insertion of genes into the *Listeria* chromosome as described herein. This plasmid contains a gram positive (for use in *Listeria)*, temperature-sensitive replication system such that growth at 37-40°C inhibits plasmid replication in *Listeria*. This plasmid also contains an *E. coli* replication system which is not temperature-sensitive (Smith et al., 1992, Biochimie 74:705-711). The plasmid, or even more temperature-sensitive derivatives thereof, may be further modified by inserting an alanine racemase gene into the plasmid, which modified plasmid is then inserted into an AA strain of *Listeria*. *Listeria* cells having the plasmid inserted

therein, are replicated at 30°C for a short period of time in order that some molecules of racemase are accumulated in the cytoplasm. The *Listeria* cells, so replicated are then injected into an animal or a human, wherein plasmid replication then ceases because of the temperature sensitive nature of the replication system at 37°C. Essentially, the cells would divide only a few times until the available racemase becomes diluted out, wherein the cells would cease replication altogether and become attenuated again. To ensure even tighter temperature sensitive replication, a temperature sensitive promoter may be used to regulate expression of the racemase gene and/or temperature sensitive mutations may be created in the racemase gene itself.

For treatment of cancer, the vaccine of the invention may be used to protect people at high risk for cancer. In addition, the vaccine may be used as an immunotherapeutic agent for the treatment of cancer following debulking of tumor growth by surgery, conventional chemotherapy, or radiation treatment. Patients receiving such treatment may be administered a vaccine which expresses a desired tumor antigen for the purpose of generating a CTL response against any residual tumor cells in the individual. The vaccine of the present invention may also be used to inhibit the growth of any previously established tumors in a human by either eliciting a CTL response directed against the tumor cells *per se*, or by eliciting a CTL response against cells which synthesize tumor promoting factors, wherein such a CTL response serves to kill those cells thereby diminishing or ablating the growth of the tumor.

The vaccine of the invention may be maintained in storage until use. Storage may comprise freezing the vaccine, or maintaining the vaccine at 4°C, room temperature, or the vaccine may first be lyophilized and then stored.

The invention particularly contemplates administration of a vaccine to a human for the purpose of preventing, alleviating, or ablating HIV infection. The protocol which is described herein for the administration of a vaccine to a human for the purpose of treating HIV infection is provided as an example of how to administer an attenuated auxotrophic *Listeria* strain as a vaccine to a human. This protocol should not be construed as being the only protocol which can be used, but rather, should be

construed merely as an example of the same. Other protocols will become apparent to those skilled in the art when in possession of the present invention.

Essentially, an auxotrophic strain of *L. monocytogenes* which requires D-alanine for growth is constructed as described in the examples. The mutant is constructed by generating deletion mutations in both the *dal* gene and the *dat* gene, essentially following the procedures of Camilli et al., (1993, Mol. Microbiol. 8:143-157). The mutant strain is then modified using recombinant DNA techniques to express an HIV-1 antigen, preferably an antigenic portion of the gag protein, essentially as described in Frankel et al. (1995, J. Immunol. 155:4775-4778). A human is then immunized by injecting a solution containing the auxotrophic *L. monocytogenes* strain and a supplement of D-alanine.

One of ordinary skill in the art will know the quatities of cells and D-alanine which should be administered to the human based upon a knowledge of the dosages provided herein which are administered to mice. For example, in BALB/c mice, 10^7 cells and 20 mg of D-alanine are the preferred dosages. Subsequent injections of the modified *L. monocytogenes* cells and D-alanine may also be given to boost the immune response.

Other HIV-1 antigens or proteins that may be used to generate a vaccine in accordance with this invention are the HIV env protein or its component parts, gp120 and gp 41, HIV gag, HIV nef and HIV pol or its component parts, reverse transcriptase and protease.

Isolated nucleic acid sequences encoding the *dal* gene and the *dat* gene of *L. monocytogenes* are also contemplated as part of this invention. In addition to their utility in generating deletion mutants of *L. monocytogenes* as disclosed herein, these isolated nucleic acid sequences encoding the *dal* gene and the *dat* gene may be used as probes and primers in identifying homologous genes in other *Listeria* species using PCR and other hybridization technology available in the art and described, for example, in Sambrook, et al. (1989, In: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York; Innis et al., ed., 1990, In: PCR Protocols,

Academic Press, Inc., San Diego). Additionally, the isolated nucleic acid sequences encoding *dal* or *dat* may be used to construct a suicide plasmid that expresses one or both of the genes. The suicide plasmid(s) may be used to complement the D-alanine *Listeria* auxotrophs for a limited time after immunization as disclosed herein.

An "isolated nucleic acid", as used herein, refers to a nucleic acid sequence, a DNA or an RNA or fragment thereof which has been separated from the sequences which flank it in a naturally occurring state, *e.g.*, a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, *e.g.*, RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g, as a cDNA or a genomic or cDNA fragment produced by PCR amplification, restriction enzyme digestion or chemical synthesis) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

Typically probes and primers for use in identifying other *dal* and *dat* genes will comprise a portion of a *Listeria dal* or *dat* gene that is at least about 15 consecutive nucleotides. More typically, a probe or primer comprises a portion of at least about 20, even more typically, at least about 30 and even more typically, at least about 40 consecutive nucleotides of a *dal* or *dat* gene of *Listeria.*

In other related aspects, the invention includes a vectors which comprises an isolated nucleic acid encoding *dal* or *dat* and which is preferably capable of directing expression of the protein encoded by the nucleic acid in a vector-containing cell. The invention further includes cells comprising a vector encoding *dal* or *dat,* including both prokaryotic and eukaryotic cells.

The isolated nucleic acids of the invention should be construed to include an RNA or a DNA sequence specifying the *dal* gene or the *dat* gene, and any modified forms thereof, including chemical modifications of the DNA or RNA which render the nucleotide sequence more stable when it is cell free or when it is associated with a cell. Chemical modifications of nucleotides may also be used to enhance the efficiency with which a nucleotide sequence is taken up by a cell or the efficiency with which it is expressed in a cell. Any and all combinations of modifications of the nucleotide sequences are contemplated in the present invention.

The invention should not be construed as being limited solely to the DNA and amino acid sequences shown in Figures 1 and 3. Once armed with the present invention, it is readily apparent to one skilled in the art that any other DNA and encoded amino acid sequence of the *dal* and *dat* genes of other *Listeria* species may be obtained by following the procedures described herein. The invention should therefore be construed to include any and all *dal* and *dat* DNA sequence and corresponding amino acid sequence, having substantial homology to the *dal* and *dat* DNA sequence, and the corresponding amino acid sequence, shown in Figures 1 and 3, respectively. Preferably, DNA which is substantially homologous is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous and most preferably about 90% homologous to the *dal* or *dat* DNA sequence shown in Figures 1 and 3, respectively. Preferably, an amino acid sequence which is substantially homologous is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous and most preferably about 90% homologous to the amino acid sequences encoded by the *dal* and *dat* genes shown in Figures 1 and 3, respectively.

"Homologous" as used herein, refers to the subunit sequence similarity between two polymeric molecules, *e.g.*, between two nucleic acid molecules, *e.g.*, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then

they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, *e.g.*, 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGCG5' share 50% homology.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

The experimental examples described herein provide procedures and results which establish that attenuated auxotrophic mutants of *L. monocytogenes* are useful as vaccines for eliciting a CTL response.

Materials and Methods useful in the construction and use of an attenuated auxotrophic *L. monocytogenes* strain are now described.

Bacteria and plasmids. The *L. monocytogenes* strain 10403S (Portnoy et al., 1988, *supra*) comprises the wild-type organism used in these studies. This organism was propagated in brain/heart infusion medium (BHI) (Difco Labs). *L. monocytogenes* strain 10403S has an LD_{50} of approximately 3 X 10^4 when injected intravenously or intraperitoneally into BALB/c mice (Schafer et al., 1992, J. Immunol. 149:53-59).

E. coli DH5a was used for cloning. This organism was propagated in Luria broth (Sambrook et al., 1989, *supra*). The plasmid pKSV7, which was used for allelic exchange reactions in *L. monocytogenes*, is a shuttle vector capable of replication in *E. coli*, where it is selected in the presence of 50 μg of ampicillin per ml of media, and in *L. monocytogenes*, wherein replication of the plasmid is temperature

sensitive and is selected in the presence of 10 μg of chloramphenicol per ml of media (Smith et al., 1992, Biochimie 74:705-711). Plasmid DNA obtained from *E coli* and total DNA (chromosomal and plasmid) from obtained from *Listeria monocytogenes* were isolated using standard methods (Sambrook et al., 1989, *supra*).

Identification of D-alanine synthesis genes in *L. monocytogenes* by homology with D-alanine synthesis genes in other gram positive organisms. Based on sequences of the alanine racemase gene (*dal*) in gram-positive organisms (Ferrari et al., 1985, Bio/technology 3:1003-1007; Tanizawa et al., 1988, Biochemistry 27:1311-1316), primers were designed which corresponded to two 20 base consensus sequences from highly conserved regions at the 5' and 3' ends of the *dal* gene. These primers were modified to reflect the preferred codon usage in *Listeria*. These primers were used in a PCR reaction using chromosomal DNA from either *L. monocytogenes* or *B. subtilis* as templates. A similar sized PCR product (850 nucleotides) was obtained from both *L. monocytogenes* and *B. subtilis*. Analysis of the 850 nucleotide PCR product from the *Listeria* template, and the amino acid sequence encoded thereby, indicated substantial homology with the alanine racemase genes of the other gram-positive organisms.

A similar strategy was used to identify and sequence a portion of a D-amino acid aminotransferase gene (*dat*) of *Listeria*, based on sequences in *B. sphaericus*, *B. species* YM-1 (Tanizawa et al., 1989, *supra*), and Pucci et al., 1995, J. Bacteriol. 177:336-342). Primers based on *dat* sequence in the other gram positive organisms was used for PCR amplification of *L. monocytogenes* DNA and a PCR product of about 400 nucleotides was obtained. Analysis of the DNA sequence of the 400 nucleotide PCR product, and the amino acid sequence encoded thereby, indicated substantial homology with the aminotransferase genes of the other gram positive organisms.

Strategy for sequence determination of the complete genes. The sequence of the remaining portions of the *L. monocytogenes dal* gene adjoined to the 5' and 3' ends of the central PCR product was determined using anchored PCR reactions

(Rubin et al., 1993, Proc. Natl. Acad. Sci. USA 90:9280-9284). Briefly, this procedure utilized a *Bgl*II-restriction digest (for the 5' portion of the gene) or a *Xba*I digest (for the 3' portion of the gene) of *Listeria* chromosomal DNA. The ends of the digested *Listeria* chromosomal DNA were then ligated to a small fragment of DNA containing the T7 promoter. A 5'-portion PCR product and a 3'-portion PCR product were then made and sequenced using primers from within the central *dal* gene PCR product and a second primer homologous to the T7 promoter fragment. This procedure permitted determination of the entire sequence of the *dal* gene.

The sequence of the remainder of the *dat* gene was determined by use of an inverse PCR reaction (Collins et al., 1984, Proc. Natl. Acad. Sci. USA 81:6812-6816; Triglia et al., 1988, Nucl. Acids Res. 16:8186). Briefly, a *Hind*III digest of *Listeria* chromosomal DNA was permitted to self-ligate under conditions of low DNA concentration so that mainly single circular molecules would form. Outward-directing primers with homologies at the two ends of the original PCR segment of the gene were then used to make a new PCR product that began at the 5'-end of the original PCR segment, proceeded to the 5'-end of the gene through the *Hind*III self-ligation site and into the 3' - end of the gene. Using this method, the entire *dat* gene sequence was obtained.

Production of mutations in *Listeria dal* and *dat* genes. The *dal* gene was inactivated by means of a double allelic exchange reaction following the protocol of Camilli et al. (Camilli et al., 1993, Mol. Microbiol 8:143-157). A *ts* shuttle plasmid pKSV7 (Smith et al., 1992, *supra*) construct containing an erythromycin gene (Shaw and Clewell, 1985, J. Bacteriol. 164:782-796) situated between a 450-base pair fragment of the 5' end of the 850-base pair *dal* gene PCR product and a 450-base pair fragment of the 3' end of the *dal* gene PCR product was introduced into *Listeria* to produce a double allelic exchange reaction between the chromosomal *dal* gene and the plasmid pKSV7 *dal* construct. A *dal* deletion mutant covering about 25% of the gene in the region of its active site was obtained.

The chromosomal *dat* gene of *L. monocytogenes* was also inactivated using a double allelic exchange reaction. A pKSV7 plasmid construct containing 450-base pair fragments corresponding to the 5' and 3' ends of the *dat* gene PCR product, which had been joined together by an appropriate PCR reaction, was introduced into *Listeria*. A double allelic exchange reaction between the chromosomal *dat* gene and the *dat* plasmid construct resulted in the deletion of 30% of the central bases of the *dat* gene.

Infection of Cells in Culture. To examine the intracellular growth of the attenuated strain of *Listeria* in cell culture, monolayers of J774 cells, a murine macrophage-like cell line, primary murine bone marrow macrophages, and the human HeLa cell line, were grown on glass coverslips and infected as described (Portnoy et al., 1988, *supra*). To enhance the efficiency of infection of HeLa cells, a naturally non-phagocytic cell line, the added bacteria were centrifuged onto the HeLa cells at 543 x g for 15 minutes. At various times after infection, samples of the cultures were obtained in order to perform differential staining for the determination of viable intracellular bacteria, or for immunohistochemical analysis.

Immunohistochemistry. Coverslips with attached infected macrophages or HeLa cells were washed with PBS, and the cells were fixed in 3.2% formalin and permeabilized using 0.05% Tween 20. *Listeria* were detected using rabbit anti-*Listeria* O antiserum (Difco Laboratories) followed by LSRSC-labeled donkey anti-rabbit antibodies or coumarin-labeled goat anti-rabbit antibodies. Actin was detected using FITC- or TRITC-labeled phalloidin. To distinguish extracellular (or phagosomal) from intracytoplasmic bacteria, the former were stained prior to permeabilization of the cells.

Induction of listeriolysin O-specific CTLs. Female BALB/c mice, 6 to 8 weeks of age (Charles River Laboratories, Raleigh, NC) were immunized by intraperitoneal inoculation with either wild-type or *dal dat* strains of *L. monocytogenes*. After 14 days, some of the mice were boosted with a second inoculation containing the same number of microorganisms as were given in the first inoculation. Ten or more days after the last inoculation, 6×10^7 splenocytes obtained

from a given animal were incubated in Iscove's modified DMEM with 3 x 10^7 splenocytes from that same animal that had been loaded with 10 μM listeriolysin O (LLO) peptide 91-99 during a 60 minute incubation at 37°C. After five days of *in vitro* stimulation, the resulting cultures were assayed for the presence of CTL activity capable of recognizing LLO-peptide-labeled P815 cells following previously published procedures (Wipke et al., 1993, Eur. J. Immunol. 23:2005-2010; Frankel et al., 1995, *supra*). Every determination of lytic activity was corrected for activity in unlabeled target cells, which exhibited between 1 and 10 percent lysis.

Animal protection studies. Female BALB/c mice (Bantin-Klingman, Freemont, CA) at 8 weeks of age were immunized with approximately 0.1 LD_{50} of viable wild-type *L. monocytogenes* or the *dal dat* double mutant strain in 0.2 ml of vehicle, by tail vein injection. Three to four weeks following immunization, groups of four to five mice each were challenged with approximately 10 LD_{50} of viable wild-type *L. monocytogenes* strain 10403 in 0.2 ml of vehicle, by tail vein injection. Spleens were removed from the mice 48 hours later and were homogenized individually in 4.5 ml PBS-1% proteose-peptone using a tissue homogenizer (Tekmar). The homogenates were serially diluted and plated onto BHI agar. Log_{10} protection was determined by subtracting the mean of the log_{10} CFU/spleen values of the test group from the mean of the log_{10} CFU/spleen values of the normal control group.

Construction of an Auxotrophic Attenuated Strain of *L. monocytogenes* Useful as a Vaccine: Construction of an attenuated strain of *L. monocytogenes* defective in cell wall synthesis

L. monocytogenes was examined to determine whether the bacteria harbor genes for the synthesis of D-alanine. The alanine racemase (*dal*) gene, used by many microorganisms for the synthesis of D-alanine, has been sequenced in *Salmonella* (Galakatos et al., 1986, Biochemistry 25:3255-3260; Wasserman et al., 1984, Biochemistry 23:5182-5187), *B. subtilis* (Ferrari et al., 1985, Bio/technology 3:1003-1007), and *B. stearothermophilis* (Tanizawa et al., 1988, Biochemistry 27:1311-1316), but the gene has not been reported in *Listeria*. Primers based on the

sequences (adjusted for preferred codon usage in *Listeria*) of two highly conserved regions of the *dal* gene in two different gram-positive organisms were employed in a PCR reaction performed on *L. monocytogenes* chromosomal DNA to search for evidence of the *dal* gene in *Listeria*. A product that exhibited significant homology with the published *dal* gene sequences was obtained. The sequence of the remainder of the *L. monocytogenes dal* gene was determined as described herein and is depicted in Figure 1. The translated protein sequence is compared with alanine racemases of the other gram-positive organisms in Figure 2.

The *dal* gene was inactivated by an in-frame insertion of a 1.35 kb fragment of DNA encoding erythromycin resistance at an Spe1 site near the center of the gene. The resulting *dal*⁻ bacteria were found to grow both in rich bacteriological medium (BHI) as well as in a synthetic medium in the presence or absence of D-alanine. Mutation of the *dal* gene was also achieved by an in-frame deletion covering 82% of the gene with the same effect.

A second enzyme used by some bacteria for synthesis of D-alanine is D-amino acid aminotransferase, encoded by the *dat* gene (Tanizawa et al., 1989, J. Biol. Chem. 264:2450-2454; Pucci et al., 1995, J. Bacteriol. 177:336-342). Following the same strategy used to detect the *dal* gene in *L. monocytogenes*, a PCR product that exhibited significant sequence homology with known *dat* genes and gene products was obtained. The sequence obtained from the PCR product was only the partial gene sequence, and remainder of the *dat* gene gene sequence (as depicted in Figure 3) was determined according to procedures described herein. The deduced protein sequence of the *L. monocytogenes dat* gene is compared with other *dat* gene products in Figure 4.

The *L. monocytogenes dat* gene was inactivated by in-frame deletion of 31% of its central region. The growth of the resulting *dat*⁻ bacteria in various bacteriological media was again found to be independent of the presence of D-alanine.

A double mutant strain of *L. monocytogenes*, *dal*⁻*dat*⁻*-1*, was produced by a double allelic exchange reaction between the erythromycin-resistant *dal*⁻ organism and the shuttle vector carrying the *dat* gene deletion. The growth of the double mutant

in bacteriological media was found to be completely dependent on the presence of D-alanine (Figure 5). A double mutant containing deletions in both of the genes, designated *dal dat-12*, had the same phenotype. The growth of the double-deletion strain in the absence of D-alanine could be complemented by a plasmid carrying the *dal* gene of *B. subtillis*. All of the *dal dat* double mutant experiments reported in the following examples employed the *dal dat-1* double mutant.

Expression of the defective phenotype following infection of eukaryotic cells

To determine whether a defect in the ability of *L. monocytogenes* to synthesize D-alanine would be expressed as an inability to replicate in the cytoplasm of eukaryotic cells because of the absence of the required D-alanine in the cytoplasm, several different cell lines and primary cells in culture were infected with the wild-type and mutant strains of this organism.

J774 cells are a mouse macrophage-like cell line that readily take up *L. monocytogenes* by phagocytosis and permit its cytoplasmic growth following escape of the bacteria from the phagolysosome (Tilney et al., 1989, J. Cell Biol. 109:1597-1608). Figure 6 depicts typical J774 cells as observed at 5 hours after infection with about 5 bacteria per cell of either wild-type *Listeria* (Panel A) or the double *dal dat* mutant *Listeria* (Panel B). Whereas large numbers of bacteria were observed to be associated with mouse cells infected with wild-type *Listeria*, few were seen following infection with the double mutant bacteria. Infection by double mutant bacteria in culture medium containing D-alainine permitted bacterial growth which was indistinguishable from that seen in cells infected with wild type *Listeria* (Figure 6, Panel C).

Some J774 cells contained small round darkly-staining objects, often in pairs, that may be spheroblast-like bacteria, although they were not examined further. When these cells were infected at higher multiplicities (a multiplicity of infection of about 1-10), many cells contained multiple microorganisms, but the double mutant again failed to multiply. Most double mutant-infected cells possessed pychnotic nuclei

and a pale cytoplasm and presumably were dead; mouse cells harboring wild-type *Listeria* did not exhibit this property at any time after infection.

To quantify some of these observations, the number of intracellular bacteria (defined by gentamicin resistance) that could form colonies on medium containing D-alanine was enumerated at several times after infection (Figure 7). The data clearly demonstrate that the double mutant was unable to replicate in J774 cells, and in fact slowly died during the course of the experiment. The data also illustrate that the replication-defective phenotype of the double mutant was supressed by the inclusion of D-alanine (at 100 μg/ml)in the tissue culture medium at the time of infection. This suppression was reversed within 2 hours after removal of the D-alanine. The phenotype of the mutant bacteria was also examined in mouse bone marrow-derived macrophages and in the HeLa cell line of human epithelaial cells. It was determined that the double mutant was unable to replicate in either of these cell types as well (Figure 7, Panels B and C).

It was again observed that double-mutant-infected macrophages possessed pychnotic nuclei more frequently than did macrophages infected with wild-type bacteria. Infection of bone marrow macrophages was employed to examine the intracytoplasmic status of the bacteria. Within a few hours after infection of cells by *L. monocytogenes,* when the bacteria have escaped from the phagosome, host actin filaments form a dense cloud around the intracytoplasmic bacteria, and then rearrange to form a polarized comet tail which propels the bacteria through the cytoplasm (Tilney et al., 1989, *supra*). The actin can readily be visualized using appropriately labeled anti-*Listeria* antibodies. At 2 hours post-infection using a multiplicity of infection of about 5 bacteria per cell, 25% of wild type bacteria associated with J774 macrophages were surrounded with a halo of stained actin (Figure 8, Panel A), and at 5 hours, virtually 100% of infected cells exhibited actin staining, some cells having long actin tails (Figure 8, Panel B). However, the staining of actin in double-mutant infected macrophages was much rarer (less than 2%) when compared with wild type infected cells. Nevertherless, if D-alanine was present during only the 30 minute period of

bacterial adsorption; at 2 hours post-infection ' 22% of the mutant cell-associated bacteria were surrounded with actin (Figure 8, Panel C); at 5 hours, this number of intracytoplasmic bacteria had risen to only 27% (Figure 8, Panel D). If D-alanine was present during the entire infection period (Figure 8, Panel E), the result observed in these cells at 5 hours was indistinguishable from those observed in wild type infected cells.

Since J774 cells have long been culture adapted and reflect very few of the normal properties of tissue macrophages, the entry of mutant bacteria into the cytosol of primary bone marrow macrophages which had been in culture for only 6 days was examined. Because these cells demonstrate the high bacterial killing capacity of normal macrophages, they were infected at a ratio of about 50 bacteria per cell. Under these conditions, at 2 hours after infection, 6.8% of the double mutant bacteria were found to be associated with actin in these cells, and this number increased to the same level as that observed after wild type infection (19%) by the inclusion of D-alanine for the first 30 minutes of the infection (18.2%) or for the entire period of infection (19.4%). Therefore, depending on the cell type examined, mutant bacteria in the absence of D-alanine either exhibited a very low or moderate efficiency of entering the host cytosol, or exhibited reduced binding of actin onto their surface. However, the brief presence of D-alanine during the initial phase of infection allowed a normal fraction of bacteria to enter the cytosol and bind actin.

Induction of an immune response using the attenuated bacteria

Infection of mice with *L. monocytogenes* produces a long-lived state of specific immunologic memory that enables the infected host to resist lethal challenge by the same organism for months following the primary infection. To determine whether infection of mice with sub-lethal doses of the double mutant could induce this important long lasting state of immunity, the following experiments were performed.

Mice were injected intravenously with 2×10^7 (<0.05 LD_{50}) of the double mutant and were challenged 3 to 4 weeks later with 10 LD_{50} of wild type *L. monocytogenes*. D-alanine (20 mg) was provided in the initial inoculum of mutant

organisms to be certain that the organisms were fully viable at the time of initial infection (this had the effect of reducing the LD_{50} about 10 fold). The data presented in Figure 9 demonstrate that the level of antilisterial protection was approximately 3 $\log_{10}$ following a single infection by the mutant bacteria, a similar level of protection to that generated by immunization with the wild-type organism. The same dose of mutant bacteria injected without D-alanine provided little protection.

To determine whether the high degree of protection generated by the mutant bacteria could be accounted for by their survival and replication in the infected mice, the spleens of infected animals were removed and the number of surviving mutant bacteria was assessed. In Figure 10 there is shown evidence which indicates that in the absence of D-alanine, few mutant organisms survived for more than one day after infection; the presence of D-alanine in the initial inoculum permitted a few bacteria to survive longer. Importantly, the almost complete protection obtained using mutant bacteria occurred in spite of the fact that by 2 days post-infection more than 100-fold fewer bacteria were detected in the spleens of mutant infected mice compared with wild type infected animals.

Listerolysin O peptide 91-99 is the major epitope of the listerolysin O protein and one of the major epitopes to which mice respond when mounting a cell mediated immune response against infection with *L. monocytogenes* (Bouwer et al., 1996, Infect. Immun. 64:2515-2522; Harty et al., 1992, J. Exp. Med. 175:1531-1538; Pamer et al., 1991, Nature 353:852-855). To determine whether the protective immunity generated by infection with the *dal*$^-$*dat*$^-$ double mutant strain of *L. monocytogenes* was associated with the induction of cytolytic T cells, splenocytes obtained from infected animals were assayed for their ability to lyse target cells loaded with this peptide. In Figure 11 there is shown the fact that animals that were infected intraperitoneally with 3×10^7 and were provided D-alanine subcutaneously both before and after infection exhibited strong CTL responses directed against the LLO peptide. Likewise, mice provided with D-alanine in their drinking water before and after infection mounted a modest CTL response after single infection with 3×10^7 mutant

bacteria. In the absence of D-alanine, animals infected with and boosted one time with 3×10^7 bacteria, also exhibited a modest CTL response to LLO peptide 91-99. Single infection with 3×10^7 of the double mutant bacteria in the absence of D-alanine produced no significant response (Figure 11).

The disclosures of each and every publication, patent, and patent application cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.